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Original Article

IN VITRO ANTHELMINTIC EFFICACY OF *ALPINIA NIGRA* AND ITS BIOACTIVE COMPOUND, ASTRAGALIN AGAINST *FASCIOLOPSIS BUSKI*

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ABSTRACT

Objective: *Alpinia nigra* (Zingiberaceae) is a traditionally used medicinal plant of North-east India against helminthiases. In view of its medicinal property the present study was designed to see the anthelmintic property of its major bioactive compound astragalin, compared to crude extract and praziquantel against *Fasciolopsis buski*.

Methods: Parasites were exposed to 2, 5, 10 and 20 mg/ml concentrations of plant extract and 0.025, 0.05 and 0.10 mg/ml concentration of astragalin in phosphate buffer saline. Paralyzed parasites were processed for histochemical, biochemical and morphological studies. Two important tegumental enzymes acid-and alkaline phosphatase were studies for its alterations in kinetic parameters on exposure to extracts and astragalin.

Results: Treated parasites revealed complete paralysis followed by death in a dose-dependent manner. Histochemical and biochemical studies on enzymes showed alterations in the activities. Morphologically, destruction and deformation were seen in the surface architecture of *F. buski* on exposure to different treatment. Acid-and alkaline phosphatase enzyme showed optimum activity at pH 6 and 9.5 respectively. The *Vmax* changed from 11.02 and 10.24 µM/min/mg protein in control to as low as 6.98 and 6.54 µM/min/mg tissue proteins in treated parasite for acid and alkaline phosphatase, respectively. Alterations in the *Km* values have also been noticed between the control and treated parasites.

Conclusion: The altered enzyme activities, tegumental architecture and kinetic parameters as observed under the influence of the plant extract and astragalin clearly indicate that the botanicals of the tested plant and its bioactive glycoside, astragalin acts as an anthelmintic agent against the tested fluke *F. buski*.

Keywords: Anthelmintic, *Alpinia nigra*, Astragalin, Tegumental, Kinetics.

INTRODUCTION

Helminth parasite infections are one among the major global problems with serious social and economic impact in the third world countries including India. Global estimates have figured out that billions of people all over the world are infected by helminth parasites [1, 2]. The diseases due to helminths causes extensive debasement to the health status of animals particularly grazing livestock and hamper in the livelihood of marginal farmers in terms loss of production through mortality, weight loss, reduced milk, meat, wool production etc. [3-5]. Different commercial anthelmintic drugs are in use to control helminth infections which provide substantial benefits to the livestock producers. Although anthelmintic drugs are effective for controlling and treatment of many helminthic infections, complete reliability on those drugs has reduced drastically because of its failures and in-effectiveness in present days. The continuous and semi-permanent dependency on a small range of anthelmintic compounds has led to the evolution of drug resistance in many helminth strains [5]. In addition, commercial drugs were seen to induce several side effects in host organisms such as epigastric pain, diarrhea, nausea, vomiting, headache, dizziness, edema, rashes and urticaria [6, 7]. These reasons leads to look forward for developing new drugs based on traditional knowledge and traditionally used medicinal plants as an alternative remedies [8, 9].

In recent times, there has been an increasing interest towards ethno-medical and ethno-veterinary practices across the world especially the use of medicinal plants in treating various ailments including helminthic infections. Several such studies have established the potential anthelmintic efficacy of different plant extracts *in vitro* and *in vivo* on nematodes, cestodes and trematodes [10-15]. *Alpinia nigra* is one such traditionally used medicinal plant the aqueous shoot-extract of which is consumed by the tribes of North-East India to cure helminth infections. In our previous experiments, we observed anthelmintic properties of the ethanolic shoot extract of *A. nigra* [16, 17]. Changes in the activities of various metabolic enzymes like lactate dehydrogenase, malate dehydrogenase, phosphoenolpyruvate carboxy kinase, pyruvate kinase, acetylcholinesterase etc. have been observed in *Fasciolopsis buski* on exposed to the crude extract of the plant [18]. Prominent surface topographical changes as well as ultra structural changes have also been observed once the parasites were treated with the plant extract [19, 20]. Therefore, because of its anthelmintic potential, the present study aimed to screen the anthelmintic efficacy of ethanol extract of *A. nigra* and astragalin, a flavone glycoside reported to be present in *A. nigra* [21].

MATERIALS AND METHODS

Preparation of ethanolic crude extract of *A. nigra* and collection of test parasites

Collection of plant material and preparation of ethanolic crude extract was carried out as described earlier [16]. Briefly, fresh edible shoots of *A. nigra* were collected from Tripura India. After washing, the shoots were air-dried, grounded into powder form, soaked in 90% ethanol for 3 to 4 d and refluxed in the same solvent for 12 h at 60 °C. After refluxed, the solution obtained was filtered through Whatman filter paper no. 1 and the solvent of the filtrate were evaporated to dryness at 40 °C using the rotary evaporator. Recovered dry extract was stored at 4 °C and used as crude alcoholic extract. *Astragalin* (AST), a bioactive glycoside reported to be present in the *A. nigra* was purchased from Sigma. Live and mature *F. buski* were collected in phosphate buffered saline (PBS, pH 7.4) from the intestine of the freshly slaughtered pig from the local abattoir in Mawlai, shillong and brought to the laboratory for further experimentations. Praziquantel (PZQ) (Chandra Bhagat Pharma Pvt. Ltd., India) has been used as a reference drug.

In-vitro motility, mortality, histochemical and biochemical studies

Live adult *F. buski* were incubated at 37±1ºC in 2, 5, 10 and 20 mg/ml concentrations of plants extract using 0.1% dimethyl

sulfoxide (DMSO) as a solvent and astragalin at 0.025, 0.05 and 0.10 mg/ml. concentrations. Control parasites were incubated in PBS having 0.1% DMSO only. Three replicates for each set of incubation medium were used and the time taken for attaining a paralytic state as well as death was recorded. The parasites incubated in 20 mg of plant extract were selected for histo chemical, biochemical and scanning electron microscopic studies because of the early effects of the dose compared to other concentrations.

Histochemical detection of two tegumental enzymes namely acid phosphatase (AcPase) and alkaline phosphatase (AlkPase) was done following the method of Pearse [22], using sodium β-phospho glycerate as substrate. Biochemical enzyme assay of both the enzymes were measured by estimating the p-nitrophenol formation following the method of Plummer [23] as described earlier [16].

For SEM studies, the fixed specimens were dehydrated through an ascending grade of acetone and then air-dried in tetra methylsilane [17, 24]. After gold coating, the surface topography was viewed using a JEOL JSM 6360 scanning electron microscope (JEOL Ltd., Tokyo, Japan) operated at 15 kV.

Kinetic studies

Activities of AcPase, AlkPase and kinetic parameters were studied following the methods of Plummer [18] and Kamal *et al.* [25], Alhomida *et al.* [26] and Njoku *et al.* [27] as described earlier by Swargiary *et al.* [17]. Each of the plant extract, astragalin and praziquantel was taken in two concentration (*A. nigra* = 0.5 and 1 mg/ml, AST= 1 and 5 µM and PZQ = 25 and 50 µM) and incubated with the assay mixture to see the alteration in the enzyme activity. However, effect of pH on the AcPase enzyme activity was studied by taking the pH in the range of 3 to 7. Enzyme activity or specific enzyme activities were expressed as units/mg tissue protein (1 Unit = 1 µM of *p*-nitrophenol formed per min).

Tissue protein estimation and statistical calculation

The protein content of *F. buski* was measured following the standard method of Lowry *et al.* [28] using bovine serum albumin as a standard protein. All experiments were carried out using three replicates (n = 3). Results were represented as the±SEM (standard error of means).

RESULTS AND DISCUSSION

The anthelmintic efficacy of crude ethanol extract of *Alpinia nigra*, AST and PZQ in terms of mortality and motility of *F. buski* is presented in table 1. The result showed a dose-dependent efficacy of the plant extracts astragalin (AST) and the commercial drug PZQ against the test parasites. The ethanolic extract of *A. nigra* has been found to possess highest anthelmintic efficacy at its highest dose (20 mg/ml of PBS) causing paralysis and death taking 2.14±0.48 h and 3.94±0.26 h, respectively. Of the three concentrations of astragalin (0.025, 0.05 and 0.10 mg/ml PBS) exposed to parasite, the highest dose causes paralysis leading to death taking 7.26±0.12 h and 8.73±0.23 h, respectively. The untreated control *F. buski* showed physical activity up to 21.05±0.22 h. Similar to present findings, a dose-dependent anthelmintic property was also observed among different test helminths including *F. buski* when exposed to different concentrations of *Lysimachia ramosa* extract [29]. Ethanolic extracts of *L. ramosa* paralysed the parasite at 3.64±0.14 h and 1.20±0.12 h at doses 5 mg and 50 mg/ml of PBS, respectively. Recently, Ahmed *et al.* [30] screened anthelmintic activity of 25 medicinal plants and showed that the ethanolic crude extract of *Ananas comosus, Aloe ferox, Allium sativum, Lespedeza cuneata* and *Warburgia salutaris* have highest efficacies against *Haemonchus contortus* of sheep. Similar to our study various solvent fractions of the foliar parts of *Glinus oppositifolius* (Linn.) DC were investigated for their anthelmintic activity by Dutta *et al.* [31] where the ethyl acetate fraction was found to have the highest potency against the test parasite.

Table 1: Effect of different concentration of *Alpinia nigra* extract, astragalin and praziquantel on the motility and mortality of *F. buski*

Incubation Medium	**Concentration of plant extracts (mg/ml in PBS)**							
	20		**10**		**5**		**2**	
	P (h)	**D (h)**	**P (h)**	**M (h)**	**P (h)**	**M (h)**	**P (h)**	**M (h)**
A. nigra ethanol	2.14±0.48	3.94±0.26	3.90±0.10	5.78±0.17	5.40±0.48	8.86±0.11	8.64±0.17	10±0.31
PZQ							2.71±0.26	3.55±0.42
Astragalin (mg/ml)	**P (h)**				**M (h)**			
0.025	12.79±0.77				14.70±0.14			
0.05	11.36±0.15				13.44±0.19			
0.10	7.26±0.12				8.73±0.23			

Time of paralysis (P) and Mortality (M) were represented in hours (h), Results were expressed in±SEM, n = 3

Two of the most important tegumental enzymes namely acid phosphatase and alkaline phosphatases were studied both histo chemically and biochemically in order to see their alterations in the staining intensities/enzyme activities prior to and after the parasites were exposed to *A. nigra* crude extract, astragalin and drug. After exposure of *F. buski* to different crude extracts of *A. nigra*, AST and PZQ, a pronounced decline in the visible stain intensity was observed in both the enzymes. Both AcPase and AlkPase showed higher stain intensities in the control parasites, whereas the treated parasites showed reduced intensities (fig. 1 and 2). Astragalin, on the other hand showed the similar extent of stain intensities compared to all other treatments. Besides tegumental regions of the parasites, changes were also noticed in the muscular regions of the body in all the treated tissue sections.

However, biochemical studies have shown higher enzyme activity of AlkPase (10.63±0.02 U/mg protein) compared to AcPase (1.83±0.052 U/mg protein) in control *F. buski*. The specific activities of enzymes and its percentage inhibitions are shown in fig. 3. Exposure of *F. buski* to the crude extract of *A. nigra* and PZQ showed more than 50% reduction in AcPase activity whereas AST treated parasites showed lesser inhibition. Similarly, biochemical assays of the AlkPase have also shown reduction of enzyme activity up to the extent of 44.96% in PZQ treated parasites. *A. nigra* crude extract and AST treated parasites showed more or less similar extent of percent inhibition (fig. 2).

Effects of various kinetic parameters like substrate concentrations, pH, temperature and plant extract/astragalin etc. on both the enzyme activities were studied to see any alterations in the enzyme activities. The effects of substrate concentrations on the enzyme kinetics were presented in fig. 4. Increasing substrate concentrations increased the enzyme activity to a certain limit and then decreased slowly with increasing substrate concentrations. The maximum velocity (*Vmax*) of both the enzymes was found to be almost similar with 11.03 U/mg proteins and 10.55 U/mg proteins for AcPase and AlkPase, respectively. However, the substrate concentrations (*Km*) needed to attain their maximum velocity is different. The Km values of AcPase and AlkPase were found to be 3.60 and 1.13 mM, respectively.

The influences of temperature, time of incubation and pH on both the enzyme activities were depicted in fig. 5. In both the enzymes, highest activity was observed at about 30ºC to 40ºC (fig. 5a). However, both enzymes showed different characters in term of incubation time. At a fixed p-nitrophenyl phosphate (*p*NPP) concentration incubation of enzyme assay at about 30 min gave higher activity in AlkPase, whereas 30 to 40 min of incubation at

37ºC AcPase activity was higher compared to other incubation times. However, the optimum temperatures were found to be almost similar in both the enzymes. The apparent energy of enzyme activation was determined from Arrhenius plot and the values were found to be 59.17±0.37 KJ/mole and 42.29±0.71 KJ/mole for AcPase and AlkPase, respectively. Similarly, higher enzyme activity of AcPase and AlkPase was observed in the pH range 6.0 to 7.0 and 9.0 to 10.0, respectively (fig. 5, c and d).

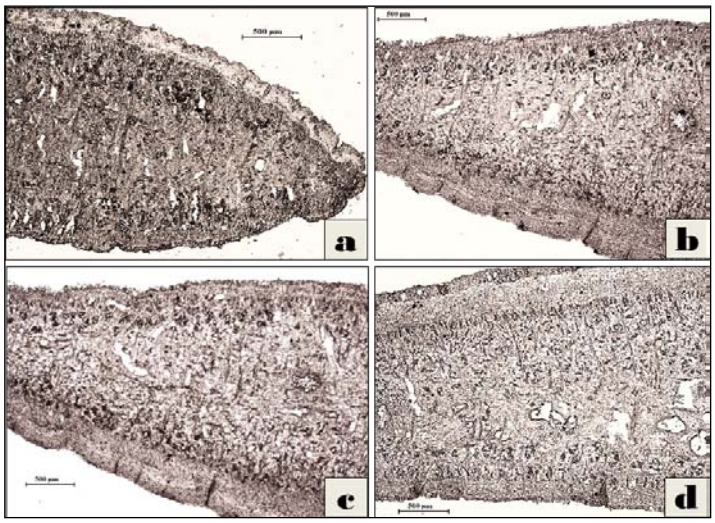

Fig. 1: Photographs of fresh frozen sections of *F. buski* showing (a) Acid phosphatase activity in control *F. buski*, (b) *A. nigra* (ethanol extract) exposed section, (c) AST exposed and (d) PZQ exposed section

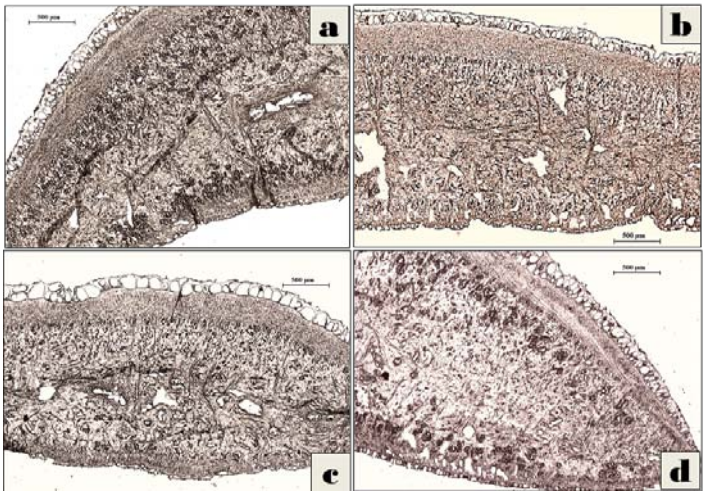

Fig. 2: Photographs of fresh frozen sections of *F. buski* showing (a) alkaline phosphatase activity in control *F. buski*, (b) *A. nigra* (ethanol extract) exposed section, (c) AST exposed and (d) PZQ exposed section

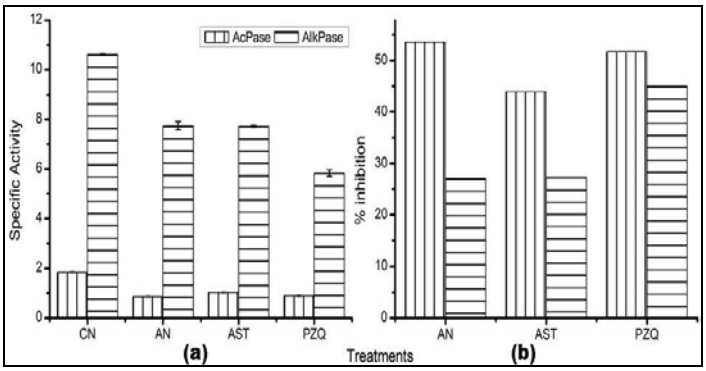

Fig. 3: (a) Enzyme activities of acid-and alkaline phosphatase in control *F. buski* and (b) percentage inhibition on treatment with the crude shoot extracts of *A. nigra* (AN), astragalin (AST) and praziquantel (PZQ) compared to untreated control (CN) *F. buski*. Values represented in±SEM

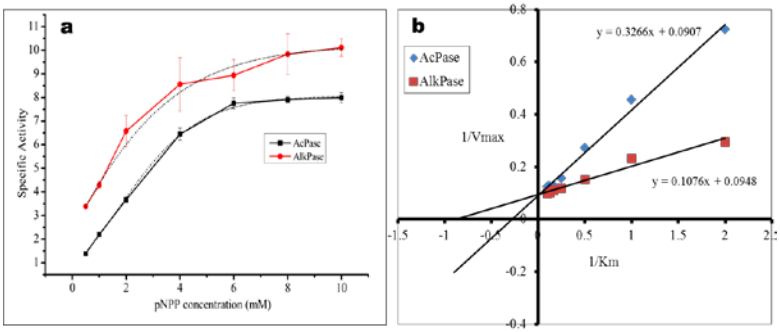

Fig. 4: Graphical representation of (a) effect of substrate concentrations on AcPase and AlkPase activity and (b) Lineweaver-Burk plot showing the *Km* and *Vmax* values of control *F. buski*

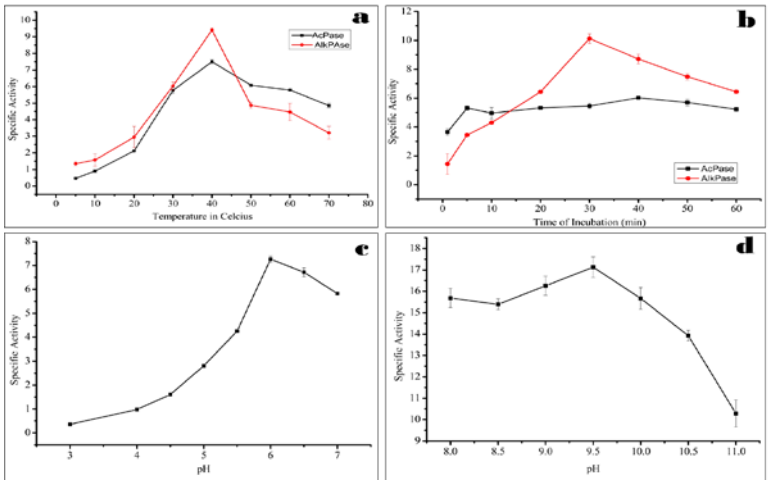

Fig. 5: Influence of temperature (a), incubation time (b) and pH on the enzyme activities of AcPase (c) and AlkPase (d) activity

The AcPase and AlkPase enzyme activities were seen to be altered on treatment with crude extract of *A. nigra*, AST and PZQ. The effects of various treatments on *p*NPP vs. enzyme activities as well as Lineweaver-Burk plots are presented in the fig. 6.

The enzyme activities (*Vmax*) of acid and alkaline phosphatase were seen to be altered after the assay mixtures were incubated with the tested concentrations of *A. nigra*, AST and PZQ (fig. 6 and table 2). On the other hand, the substrate concentrations (*Km*) to attain a maximum enzyme activity remained almost same to that of the control value 3.60 mM. It is seen from the result that the plant extracts and astragalin as well as PZQ has a mixed type of inhibition with little more tendency towards non-competitive type of enzyme inhibition in AcPase enzyme functioning.

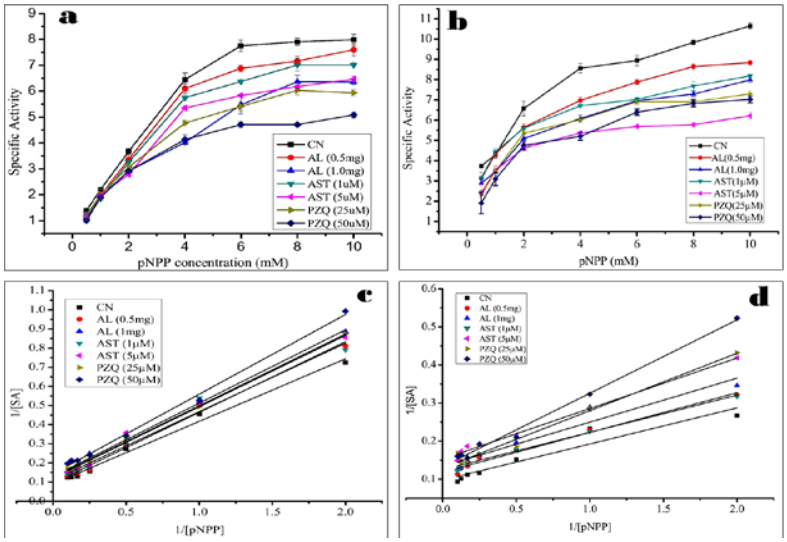

Fig. 6: Graphical representations on the effects of ethanol extract of *A. nigra* (0.5 and 1 mg/ml), astragalin (1 and 5 µM) and praziquantel (25 and 50 µM) on (a) Acid phosphatase and (b) Alkaline phosphatase activity with increasing *p*NPP concentrations, and Lineweaver-Burk plots showing altered kinetic parameters of (c) Acid-and (d) Alkaline phosphatase in the *F. buski* on treatment with the *A. nigra* (ethanol extract), astragalin and PZQ

Similar is the case with AlkPase enzyme activity, except in PZQ treated parasite where the substrate and PZQ were observed to have some kind of competition indicating a sign of competitive type of enzyme inhibition (table 2). Ethanol extract of *A. nigra* ethanol extract as well as PZQ treated parasites showed more reduced AcPase enzyme activity whereas AST showed better inhibitory activity against AlkPase.

Stereoscan observations on control *F. buski* revealed fine surface topography, with prominent scale-like papillae on the ventral surface of the body arranged in an alternate manner (fig. 7a). However, massive destruction and deformations were noticed in the surface topography of the parasites exposed to crude extract and astragalin (fig. 6b and c). Earlier Roy *et al.* [20] showed that the crude extracts of *A. nigra* and PZQ caused extreme deformation in the fine surface structures of the parasite exposed to crude extracts of *A. nigra*. In a similar kind of study, *A. oxyphylla* extract was found to cause substantial structural deformities in *R. echinobothrida* and *A. gali* [32].

Similar to the our study, treated *R. echinobothrida* parasites also exhibited extensive distortion of the surface fine topography and decrease in the activities of major tegumental enzymes compared to that of control parasite on exposed to resveratrol and virosecurinine, the active compound of Carex Species [33] and *Securinega virosa* [34], respectively. The scanning electron microscope observations of *Senna occidentalis* extract treated *Hymenolepis diminuta* showed irrevocable destruction all over the body tegument as well as sloughing of microthriches and shrinkage of scolex [35]. *In vitro* exposure of *R. echinobothrida* to α-viniferin, an active component of the plant *Carex baccans* L. also found to cause extensive distortion and disorganization of surface topography [36].

Table 2: The *Km* (mM) and *Vmax* (U/mg protein) values of acid and alkaline phosphatase on treatment with different concentrations of ethanol extract of *A. nigra*, AST and PZQ

Enzyme	Kinetic parameters	Control	*A. nigra*		Astragalin		Praziquantel	
			0.5 mg	1 mg	1 µM	5 µM	25 µM	50 µM
Acid Phosphatase	*Vmax*	11.02	10.45	7.69	9.07	8.27	8.03	6.98
	Km	3.59	3.86	2.94	3.24	3.08	3.01	2.90
Alkaline Phosphatase	*Vmax*	10.24	8.44	7.45	7.98	6.54	7.74	7.51
	Km	0.97	0.88	0.86	0.78	0.87	1.17	1.45

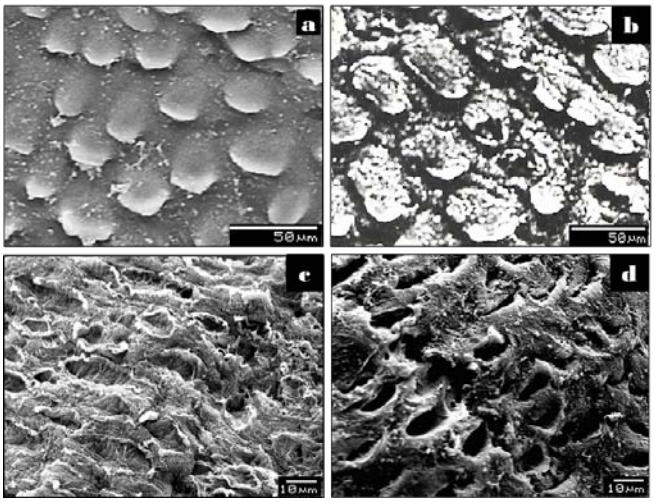

Fig. 7: Scanning electron microscope image of *F. buski* showing (a) Ventral region of the control worm with scales and denticulate projections, (b) *A. nigra* exposed worm with ventral region of body, (c) Astragalin exposed and (d) Praziquantel exposed ventral region showing sloughed scales and scars formations

CONCLUSION

In the present study, extract of *A. nigra* and its bioactive compound astragalin showed dose dependent anthelmintic effect against *F. buski*. Anthelmintic efficacy of plant bioactive compound is mediated through changes in the vital tegumental enzymes, like acid-and alkaline phosphatase, and changes in the surface ultrastructure of the parasite. These findings suggest that astragalin may prove to be an effective anthelmintic; however detailed toxicological aspects of this compound need to be studied further.

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CONFLICT OF INTERESTS

Authors declare no conflict of interest

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